Research Plan Proposal

TITLE OF THE SYNOPSIS

"Genetic diversity of selected wheat lines for rust resistance"

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Introduction

Wheat is the second most important staple food crop in world after rice as a source of calories and first as a source of protein in the diets of developing country consumers. Approximately 600 million tons of wheat is produced annually, roughly half of which is in the developing countries (Aquino et al., 2002). Wheat provides 21 % of the food calories and 20 % of the protein to the tune of more than 4.5 billion people across 94 developing countries (Braun et al., 2010). Brown rust caused serious losses in Asia, America. Globally, yellow rust assumed importance in Asia, Africa, China, America and Europe (Mc Intosh et al., 1995). Many laboratories worldwide conduct rust surveys and virulence analyses. Most currently important races have either evolved through mutations in existing populations or migrated from other, often unknown, areas (Espino, 2011).

India is leading producer of wheat (*Triticum aestivum* L.) in the world. During 2011-12, total wheat production reached all time high 93.90 million tones Economic survey, 2012-13. To increase and sustain its yield, the crop must be kept free from diseases (Prashar et al., 2015). Indian wheat programme started around 1900 and progressed towards one of the most successful in the world achieving self-sufficiency in wheat. On the way to this achievement, many important steps like breeding for higher yields and protection against rusts were taken (Tomar, 2014).

Wheat and rusts have co-existed and co-evolved hand in hand for centuries, therefore, there is a close relation between the two. India, Pakistan, Bangladesh, and Nepal grow nearly 37 mha of wheat, of which 30 million are at risk to leaf rust losses. Losses were negligible during the last 5 years due to widespread use of resistant Varieties, especially in the more rust-prone areas (Bhardwaj et al., 2013). Other steps which accompanied these activities like race identification, screening pre- released material against virulent races, using major genes and then incorporating minor gene based resistance also contributed to this successful story (Datta et al., 2007). Deployment of effective and durable resistant genes along “Puccinia path” is the most successful strategy of wheat rust management in Indian sub-continent (Kumar et al., 2015).

All the two rusts, viz. brown rust and yellow rust pose serious challenge to wheat production under favourable conditions. Losses due to three rusts though under check largely due to systemic and pre-emptive breeding, still pose a great threat as proved by incidence of yellow rust in North West plain zone. Significant losses by rusts have been reported (Asthana, 1948., Barclay, 1890., Joshi et al, 1975., Mehta, 1940., Prasada, 1960., Gupta and Singh, 1981., Sharma et al., 2011) from time to time.

Application of resistance genes in wheat is the most effective, economic and environmentally safe approach for controlling rusts (Chen et al., 2005). Rust fungi are obligate parasites and must survive on living plants. Survival during the off-season occurs on either self-sown (or voluntary) wheat plants or other grass species that can also be infected. Moreover, conditions favorable to disease development (i.e., temperature and high humidity leading to dew formation) occur most years throughout the growing season (Singh, 2004). The rusts of wheat are among the most important plant widespread pathogens that can be found in most areas of the world where wheat is grown (Cloustier et al., 2006).

Resistance is broadly classified into three categories:

1. True resistance (vertical and horizontal)
2. Non host resistance
3. Apparent resistance

Vertical resistance is controlled by one or few genes while horizontal resistance is controlled by many genes (Nayar et al., 2003).

Review of literature

Biffen (1905) was first to show that resistance to yellow rust of wheat was controlled by one recessive gene in Rivet wheat. The occurrence of different races of rusts caused considerable difference of opinion in early rust work. A cultivar resistant at one location was sometimes susceptible at other; therefore, stability of resistance was doubted. The concept became clear after the work of Stakman and co-workers at Cereal Rust Laboratory, Minnesota, the United States of America (Stakman, 1914).

In India, wheat is being grown from 2300-1750 B.C. as evidenced from the cultivation in Sind and Punjab during the Harappan period. The excavations at the ancient site of Mohan-Jo-Daro revealed the carbonized grains belonging to a sub-species of *Triticum aestivum* L (Marshall et al., 1933).

In wheat, three types of rusts occur. In temperature or cooler climate, yellow rust caused by *Puccinia striiformis* West. As temperature increases brown rust caused by *Puccinia triticina* Eriks. prevails more and, at higher temperature stem rust caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & Henn. is a major threat.

Yellow rust

Schmidt in 1927 reported first time about the fungus which caused the yellow rust of wheat in Europe, and named it as *Uredo glumarum Schmidt*. In 1894, Erikson and Hennings discussed the telia stage of the fungus and named it as *Puccinia glumarum* (Schm.) Eriks. and Hen. Later this name was reported invalid and the fungus was renamed as *Puccinia striiformis* West (Hylander et al., 1953). In India, 28 pathotypes of yellow rust (*P.striiformis* West.) namely were identified from India so far (Bhardwaj et al., 2011). The most predominate and virulent pathotypes in Northern hills and North Western plains zones of India is 46S119. In southern hill zone of India most predominant pathotypes is I is not identified in rest of India (Prashar et al., 2007).The urediospores of this fungus are nearly rounded, binucleate and unicellular (Stubbs, 1985). Occurrence of yellow rust in high severity during the last decade was due to evolution of more virulent pathotypes that were able to overcome widely used sources of resistance in wheat (Nayar et al., 1997; Prashar et al., 2007).

Brown rust

De Condolle (1815) recognized wheat leaf rust as a separate species from other rusts of wheat and described it as Uredo rubigo -vera (Chester, 1946). In 1932, Mains subdivide *P. rubigo-vera* into 56 formae species, one of which was f.sp. *tritici*, corresponded that *Puccinia recondita* was the valid name for the leaf rusts of grasses (Cummins and Caldwell, 1956). The term *P. recondite* f.sp. *tritici* was used by most, since 2002, it has been renamed as *Puccinia triticina* Eriks. uniformly by all workers. When brown rust appears very early in the crop season, it causes loss to wheat as does the stem rust (Joshi et al., 1972, 1984). Epidemics of brown rust have occurred in India from time to time such as in years of 1786, 1805, 1827, 1832, 1894, 1897, 1905, 1947, 1948, 1956, 1972 and 1973 (Nagarajan and Joshi, 1975). Mains and Jackson (1926**)** first reported the existence of physiologic races in *P.recondita*. In India, studies on the physiological of rusts were races started by K.C.Mehta in 1931 at Flowerdale, Shimla, Himachal Pradesh, India.

Management of wheat rusts

Wheat rust can be managed either by chemicals or by disease resistance in the host plant. A large number of highly effective fungicides like Propiconazole (Tilt 25EC), Tridemifon (Bayle ton 25EC) Tebuconazole (Folicur, 250WP) etc. are available, which can control the rust fungi easily (Andenow, 1988). But chemical control is not practical and economically feasible for large scale applications. On the other hand, management of wheat rust through host by the application of resistance genes in wheat is the most effective, economic, environment friendly and practical approach for controlling rusts (Chen et al., 2005).

Slow Rusting

When wheat varieties with vertical resistance are expensively grown, new pathotypes of the rust fungi consistently arise through mutation and selection, rendering these resistant varieties susceptible (Nayar et al., 1994). Wheat varieties possessing tolerance to rust and other characters for rust resistance which slow down rust development have been advocated for this purpose. Some wheat lines recognized by the slow rust development of disease and this phenomenon are known as slow rusting (Caldwell et al., 1970).

Hypothesis:-

To identify a number of differentially expressed genes involved in wheat leaf, stem and stripe rust pathosystem by host- pathogen interaction.

Objectives:-

The proposed study is designed to:

1. To identify rust resistance wheat lines.
2. Characterization of yellow, brown rust resistance genes.
3. To identify adult plant resistance genes to brown, yellow rust.
4. Identification of slow rusting wheat varieties to these some of the characterized *Lr* and *Yr* rusts of wheat.
5. Molecular validation of resistance genes with known molecular markers.

Methodology:-

All the experiments will be conducted under optimum conditions for both host and pathogen at IIWBR, RS, Flowerdale, Shimla (H.P), which is situated at 32° 29´ N latitude, 75° 10´ E longitude and has an average altitude of 2,206m above sea level. Shimla is located in the hills of Himalayas. Climate is subtropical with slightly hot and little humid summer and cold winters. The whole research work will be done with proper checks. Details of the methods are given below:-

I. Seedling resistance test-

Total approx 100 wheat cultivar will be selected for study. Raising of plant material, inoculation, recording, and characterization of rust resistance genes. Following seedling growth and inoculation methods are listed below:

1. Preparation of soil

A mixture of fine loam and farmyard manure (3:1), will be sterilized by autoclaving at 60°C for an hour, and used for sowing of plant material. Five grams of NPK (12:32:16) mixture per 5kg of soil will be used as nutrient supplement (Kumar, 2013).

2. Plant material

The seedlings will be raised in spore-proof chambers (indoors) at 22±2°C, 50- 70% relative humidity and 12-hour daylight (Kumar, 2013).

3. Multiplication of inoculum

Plastic pots (12.5cm dia) containing loam soil and farmyard manure will be used for the multiplication of inoculum. Each pot contains 15-20 plants of susceptible Agra Local, a land race. Inoculum collected after 15 days of inoculation have been used for further studies. The seedlings will be grown in aluminum bread pans (29cm long x 12cm wide x 7 cm deep size). These trays are sufficiently large to accommodate 10 lines with about 100 plants of each *Lr, Yr* gene and Agra local, susceptible check should be maintained to confirm the level of infection. Equal proportion (20mg) of each pathotypes will be mixed thoroughly and inoculated on to 300 plants of each of *Lr, Yr* genes. Separate checks of these hosts will be maintained to know the incubation period, number of uredial pustules and number of urediniospores/cm^2.

4. Recording of observations

Infection types (low or high) on the test lines should be recorded on 15 days after inoculation following Stakman et al. (1962) with some modifications. Infection types 0 to 2 (small hypersensitive flecks to small-moderate uredial pustules with chlorosis) considered low indicating resistance and infection types of 3 to 4 (moderate to large uredial pustules without chlorosis) are considered high indicating susceptibility. Infection types 33+ are classified where both 3 and 3+ pustules found together. Plant material with reaction types 0 to 2 consider for further study in molecular characterization.

5. Differential sets

Differential sets for identification as well as to check the purity level of brown and yellow rust pathotypes (Nayar et al., 2001) are given on next page:-

SET-0		SET-A		SET-B	
BROWN RUST	YELLOW RUST	BROWN RUST	YELLOW RUST	BROWN RUST	YELLOW RUST
IWP94	WH147	*Lr*14a	Chinese 166 (*Yr*1)	Loros (*Lr*2c)	Hybrid (*Sr*7b+)
Kharchia Mutant	Barley Local	*Lr*24	Lee (*Yr*7)	Webster (*Lr*2a)	Einkorn (*Sr*21+)
Raj 3765	WH416	*Lr*18	Heines Kolben (*Yr*6)	Democrat (*Lr*3a)	Kota (*Sr*28+)
PBW343	HD2329	*Lr*13	Vilmorin 23 (*Yr*3)	Thew (*Lr*20)	Reliance (*Sr*5+)
UP 2338	HD2667	*Lr*17a	Moro (*Yr*10)	Malakoff (*Lr*1)	Charter (*Sr*11+)
K 8804	PBW343	*Lr*15	Strubes Dickkopf	Benno (*Lr*26)	Khapli (*Sr*7a, *Sr*13, *Sr*14+)
Raj 1555	HS240	*Lr*10	Suwon92 X Omar	HP 1633 (*Lr*9+)	Benno (*Sr*31)
HD 2189	Anza	*Lr*19	Riebese147/51 (*Yr*9+)		
Agra Local	A-9-30-1	*Lr*28			

II. Adult plant resistance test

1. Field Experiments

This data will be used for evaluation and identification for promising wheat cultivars on the basis of low level of yellow and brown rusts infection at adult plant stage and carry out the genetic analysis. After doing these experiments, we also do some laboratory experiments for the evaluation of adult plant stage resistance through molecular techniques. Adult plant resistance may be effective against a wide-spectrum of races and add to diversity for resistance. Race-

specificity has been found for several adult plant resistance genes for brown and yellow pathogens.

Procedure is same for adult plants of different wheat lines, likewise as in seedling resistance test for preparation of soil, plant material and inoculation as well in controlled polyhouse, maintained at 22±2°C for brown rust, yellow rust15±2°C and black 24±2°C. The distance between the clumps in each row should be 22.5 cm.

2. Recording of observations

The infection types of the adult plants and seedlings can be recorded on 15 days after the inoculation as before said. Rust infection types, adult plant stage for reaction types readings are 0 (immune), R (Resistance), MR (Moderate resistance), MS (Moderate susceptible), S (susceptible) and X (Intermediate), all these recordings have been done after first appearance of the symptoms of the disease in the experimental plots.

III. *Slow rusting*

In recent years, increased awareness of the short-lived nature of vertical resistance to wheat rusts has led to more emphasis on the phenomenon of general resistance called slow rusting (Khurana, 2004). In slow rusting, mixture of most virulent and predominant pathotypes of each rust would be used and observations are recorded as per Nayar et al., 2003. Uredial pustules will be counted regularly on primary leaf after every 24 hours after the appearance of first uredial pustule till their number become constant.

Simultaneously length and breadth of the leaves will also be measured and leaf area calculated by the formula LxB÷2/3, where L and B are length and breadth of leaf respectively and 2/3 is the constant factor (Kapoor and Joshi, 1981). Latent period is calculated by the formula given below:-

$$\text{L.P.} = \frac{t_2 (F/2 - t2) - (t2 - t1)}{nt2 - nt1}$$

Where F=final number of uredial pustules, t1=day before 50% uredial pustules appeared, t2=day after 50% uredial pustules appeared, nt1= number of uredial pustules appeared on t1, nt2= number of uredial pustules appeared on t2 (Ohm and Shaner, 1976).

The population of uredial pustules per square cm on leaf surface has been found at by dividing the total number of uredial pustules on the leaf by the area of the leaf. Urediniospores per pustule will be calculated with the help of haemocytometer. Urediniospores /cm^2 have been

calculated by multiplying the number of uredial pustules /cm^2 with the number of urediniospores /pustule.

IV. *Lr* and *Yr* genes characterization

This technique has been design to test the genes of brown and yellow rust in shortest period of time. As we know, rust disease is outcome of the interaction between host and pathogen. Flor (1956) proposed the gene for gene hypothesis, which was later conceptualized by Person et al., 1962 as the interaction between the two genes which leads to a single phenotypic expression.

It is assumed by applying the gene matching technique using multipathotype data (Browder, 1973). In this characterization, the resistance genes for brown and yellow rust resistance genes could be postulated only if the differential host response is present.

V. DNA polymorphism (extraction of DNA, purification of DNA, quantification and amplification of DNA, electrophoresis)

All these techniques will be performed to confirm the resistance genes. The resistance genes incorporated in the cultivars are mostly short lived because of the continuous evolution of new pathotypes (Datta, 2006). One of the effective remedy for increasing the longevity of resistance genes is to develop varieties with more than one effective resistance gene. Through conventional techniques it is not possible to confirm the incorporation of two or more rust resistance genes in the absence of differential host pathogen interaction. However, with the aid of molecular markers it is possible to pyramid more than one resistance genes.

Molecular Characterization

DNA extractions from wheat leaves

For the evaluation of adult plant resistance, through molecular techniques the DNA from selected wheat lines will be extracted from the young seedlings. The detailed procedure is listed below:

The genomic DNA extracted from molecular characterization studies by using CTAB method of Doyle and Doyle (1990) with some modifications.

a. Genomic DNA isolation from tissues of a plant using CTAB method. DNA is a high molecular weight macromolecule. CTAB method is commonly used due to less expensive and high yield of genomic DNA.

b. Purification of DNA by RNase treatment ribonuclease A is an endonuclease enzyme, having the capacity to digest RNA.

c. Quantification and amplification of DNA

Each DNA sample will be subjected to electrophoresis in agrose gel and the quantity of DNA can be visually estimated. DNA amplification will be done by the modification of original Polymerase Chain Reaction (PCR), it is based on the enzymatic amplification of DNA fragments that is flanked with oligonucleotide primers hybridizing to opposite strands of the target sequence.

The PCR involves three basis steps which constitute a single cycle

- Denature the target DNA at 92-94°C.
- Annealing of the primers to the templates DNA.
- Primer extension by addition of nucleotides to the 3´ ends of the primers by the enzyme DNA polymerase.

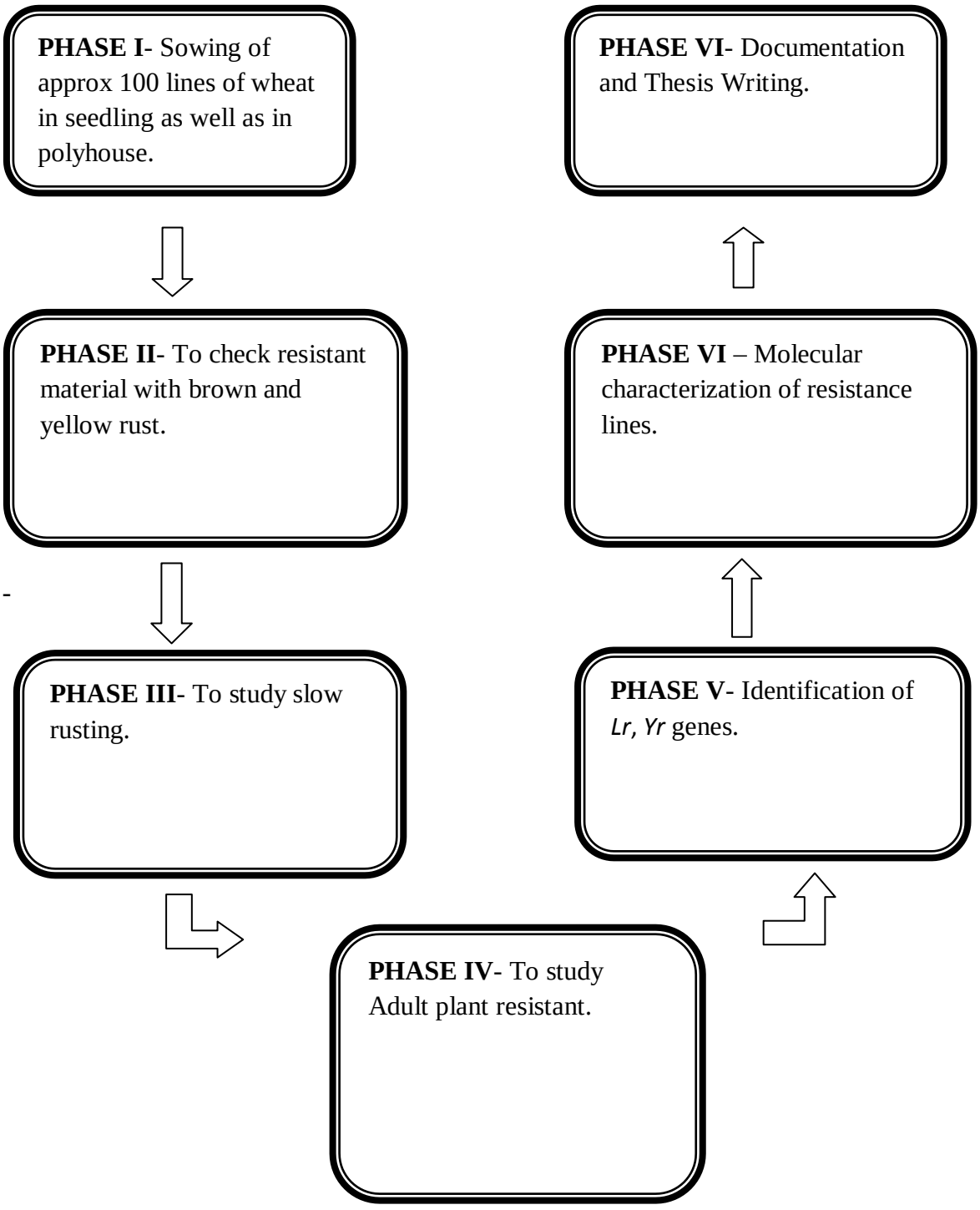
There are number of variables in a PCR which have to be optimized to give target amplification. These parameters are:

- Denaturation temperature and denaturation time.
- Annealing temperature and annealing time.
- Amounts of template DNA and primer to be taken.
- Concentration of $MgCl_2$ in the assay buffer.
- The number of cycles to be performed.

d. Electrophoresis:

After amplification, loading dye will be added to each PCR product and spinned at 2000 rpm. The bands shown in computer will be photographed under ultraviolet light in gel scanner through UV-photo computer programme.

Plan of work


PHASE I- Sowing of approx 100 lines of wheat in seedling as well as in polyhouse.
PHASE II- To check resistant material with brown and yellow rust.
PHASE III- To study slow rusting.
PHASE IV- To study Adult plant resistant.
PHASE V- Identification of *Lr, Yr* genes.
PHASE VI – Molecular characterization of resistance lines.
PHASE VI- Documentation and Thesis Writing.

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SIGNIFICANCE OF THE WORK AND FUTURE IMPLIFICATIONS

Rust evolves into new pathotypes rendering resistance genes susceptible. These are air – borne, epidemic in nature. **In the words of Dr. S. Nagarajan "Brown rust of wheat is TB, Yellow rust of wheat is Cholera"**. So here, I present a proposed scenario that will allow a quantitative and qualitative characterization of various wheat lines.

This data can be very useful to help out various leaf, stripe rust diseases. The generated data can be utilized to correlate selected genes with resistance and breed to new varieties by adding resistance genes. Further this would help us to understand the host-pathogen interaction at molecular level.

Moreover, being simple, quick and relatively easy to perform experimental work, short term practical work can assess the result out come in short period of time. In case of positive results in glasshouse and field material, from a scientific point of view, extensive monitoring programs should be performed in order to identify new genes related to resistance.

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